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The California Academy of Sciences Gulf of Guinea Expeditions (2001, 2006) VI. A New Species of *Phrynobatrachus* from the Gulf of Guinea Islands and a Reanalysis of *Phrynobatrachus dispar* and *P. feae* (Anura: Phrynobatrachidae)

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The oceanic islands of São Tomé and Príncipe in the Gulf of Guinea of Africa harbor a surprising number of endemic amphibians. Two species of puddle frogs (*Phrynobatrachus*) have been described from these islands: *Phrynobatrachus dispar* (Peters, 1870) and *P. feae* (Boulenger, 1906). The validity of *P. feae* as a taxon distinct from *P. dispar* has been in doubt and in recent works the two have been considered synonymous. However, a detailed analysis has never been performed. We examined 175 specimens of *Phrynobatrachus* collected from the two islands during the 2001 and 2006 CAS Gulf of Guinea expeditions as well as two syntypes of *P. feae*. Consistent external morphological and osteological differences were found between specimens from different islands. Furthermore, maximum likelihood analysis of cytochrome b sequences revealed a high mean inter-island sequence divergence of 21%, whereas intra-island distances were only around 1%. This level of divergence indicates an ancient split, possibly predating the formation of São Tomé. Mitochondrial DNA sequences from 12S rRNA, valine-tRNA, and 16S rRNA genes support this divergence and indicate that the *P. dispar* clade is sister to an East African clade of *Phrynobatrachus*, and not West African species, a recurrent theme with this insular amphibian fauna. Because the type localities of both currently available names are on Príncipe Island, the species endemic to São Tomé is undescribed. We thus describe a new species of *Phrynobatrachus*, raising the current total of endemic amphibian species in the Gulf of Guinea Islands from six to seven.

As ilhas oceânicas de São Tomé e Príncipe no Golfo de Guiné da Africa, abrigam um número significativo de anfibios endêmicos. Duas espécies de rãs que vivem em poças d'água (*Phrynobatrachus*) foram descritas nestas ilhas: *Phrynobatrachus dispar* (Peters, 1870) e *P. feae* (Boulenger, 1906). A validade de que *P. feae* é um taxon distinto de *P. dispar* esteve em dúvida e, em um trabalho recente, as duas espécies foram consideradas a mesma. No entanto, um detalhe na análise nunca foi executado. Nós examinamos 175 espécimes de *Phrynobatrachus* coletados nas duas ilhas, incluindo os dois syntypes de *P. feae*, durante as expedições da California Academy of Sciences no Golfo da Guiné, em 2001 e 2006. Diferenças morfológicas externas e osteológicas

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foram observadas entre os espécimes de ilhas diferentes. Além disso, a análise da probabilidade máxima das seqüências do citocromo b revelaram uma média alta de divergência inter-ilha de 21%, enquanto as distâncias intra-ilha foi ao redor de 1%. Tal nível de divergência indica uma separação muito antiga, possivelmente pré-datando a formação de São Tomé. As seqüências de ADN mitocondrial do rRNA 12S, tRNA-valina, e genes rRNA 16S confirmam esta divergência e indica que o clade *P. dispar* é proveniente do clade africano do leste de *Phrynobatrachus*, e não da espécie Africana do oeste. Considerando que as duas espécies já nomeadas estão localizadas na Ilha de Príncipe, as espécies endêmicas a São Tomé não estão devidamente representadas. Sendo assim, nós descrevemos uma espécie nova de *Phrynobatrachus*, aumentando o número total de espécies endêmicas de anfibios no Golfo das Ilhas de Guiné de seis para sete.

The oceanic islands in Gulf of Guinea archipelago contain some of the highest levels of endemicity in the world, including six currently recognized endemic amphibian species. São Tomé and Príncipe lie 280 km and 220 km off the coast of mainland Africa respectively, with ocean depths between the continent and the islands reaching 4000 m. Geologic evidence indicates these two islands and out-lying Annobon have never been connected to the mainland, which suggests dispersal as the most likely mechanism for colonization (Measey et al. 2007). Traditionally, transoceanic dispersal by amphibians is thought to be highly unlikely because of low physiological tolerances for salinity, and the presence of endemic amphibians of the Gulf of Guinea islands, including the fossorial caecilian *Schistometopum thomense,* has been among the most remarkable and perplexing biogeographic mysteries. Nevertheless, several recent studies suggest that transoceanic dispersal of amphibian species must have occurred (Fahr 1993; Vences et al. 2003; Vences et al. 2004; Measey et al. 2007).

The diminutive anurans of the genus *Phrynobatrachus* (Günther 1862) are notoriously difficult to distinguish morphologically. These species are generally highly polymorphic, quite small in size, cryptically colored, and often have limited descriptions in the literature (Stewart 1974; Drewes and Perret 2000; Hoffman and Blouin 2000; Crutsinger et al. 2004). Consequently, we understand little about this widespread genus, which is among the most speciose of African anurans.

Two species of *Phrynobatrachus* have been described (as *Arthroleptis* Smith) from the Gulf of Guinea islands of São Tomé and Príncipe: *P. dispar* (Peters 1870) and *P. feae* (Boulenger 1906). Type localities for both species are given as the island of Príncipe, although the range of *P. dispar* was later expanded to include São Tomé (Loumont 1992). Boulenger gave very few characters to define *P. feae* as a species distinct from *P. dispar.* Peters described *P. dispar* as being 20 mm snout-vent length (SVL) whereas Boulenger indicated the maximum length for 25 specimens of *P. feae* as 12 mm and 15 mm SVL for males and females respectively. Furthermore, only one character is provided in Boulenger's dichotomous key to distinguish the two species:

> …inner metatarsal tubercle considerably nearer to the outer than to the tarsal tubercle . . . . . *A. dispar*
> …inner metatarsal tubercle tubercle equally distant from the outer and from the tarsal tubercle . . . . . *A. feae*

Since then, only limited attention has been given to the Gulf of Guinea *Phrynobatrachus*. Loumont (1992) found that the spacing between tubercles was unreliable and seemed to vary with ontogeny, and that it was unlikely that two species could occupy the same niche on the small island of Príncipe. *Phrynobatrachus feae* has been treated as a junior synonym of *P. dispar* by subsequent

authors (Loumont 1992; Schätti and Loumont 1992; Drewes and Stoelting 2004; Frost 2004).

Jonathan Baillie (1999) observed *Phrynobatrachus* at all elevations on Príncipe and noticed that there seemed to be two distinct size classes: a small size class congregating by the rivers at night and a larger size class seen inhabiting the forest. He also suggested that there might be more polymorphism among Príncipe populations than in populations from São Tomé, suggesting the possible existence of two species on Príncipe. Baillie also speculated that *P. feae* may simply be juvenile *P. dispar* or, if a distinct species, that the former might be a dwarfed form of *P. dispar.*

Loumont (1992) stated that amphibians occur in two ecological zones on the islands, a low elevation zone from 0–500 m where *Phrynobatrachus was* found, and a middle montane zone from 500–1000 m. However, both Baillie (1999) and Drewes and Stoelting (2004) observed *Phrynobatrachus* at considerably higher elevations than previously reported on both São Tomé and Príncipe, including on the summit of Pico de Príncipe at 948 m and at an elevation of 1412 m on São Tomé; therefore, the possibility exists that two species of *Phrynobatrachus* might inhabit the same island by occupying different elevational zones.

For this study, we examined the largest collection of Gulf of Guinea *Phrynobatrachus* in existence. Specimens were taken at multiple elevations on both islands and analyzed using a combined dataset of morphological, osteological and molecular characters. The datasets provide compelling evidence for the recognition of two distinct species of *Phrynobatrachus*, each endemic to a single island. Because the type locality of both *P. dispar* and *P. feae* is Príncipe Island, we herein describe a new species endemic to the island of São Tomé and raise the number of recognized amphibian species from the oceanic islands in the Gulf of Guinea islands to seven.

Materials and Methods

Collection of specimens.— One hundred and fourteen individuals were collected during the California Academy of Sciences' (CAS) first Gulf of Guinea Expedition (March–June, 2001) and an additional 61 frogs were collected during the second expedition, March–June 2006. Specimens were hand collected, euthanized and preserved in 10% formalin (Drewes and Stoelting 2004). Sixty-four individuals from 10 localities were collected from São Tomé and a total of 112 individuals from 10 localities were collected from Príncipe (Fig. 1; Appendix Table 1). The sample is heavily biased toward calling males because these are easier to locate in the field. Tissue samples, taken from 25 individuals from seven localities on São Tomé and 19 individuals from four localities on Príncipe, were placed in 95% ethanol. Institutional abbreviations follow Leviton et al. (1985).

Morphology.— We measured 18 morphometric characters for 175 of the specimens (Appendix Table 1). In addition, we examined outgroup specimens of *Phrynobatrachus calcaratus* ($n = 5$), *P. parvulus* ($n = 3$), *P. cornutus* ($n = 8$) and *P. minutus* ($n = 11$). Measurements were taken using Vernier calipers, frequently with the aid of a dissection microscope, to a precision of 0.1 mm. Measurements recorded include: snout-vent length (SVL), head length (HDL), head width (HDW), width of the eye at its widest point (EYE), width of the interorbital space (IOS), width of the internarial space (INS), distance between the eye and the naris (ENS), distance between the naris and the tip of the snout (NS), distance between the naris and the edge of the upper lip (NLL), tibia-fibula length (TiL), femur length (FeL), tarsus length (TaL), distance between the inner and outer metatarsal tubercles (IOMT), distance between the inner and tarsal tubercles (ITT), distance between the tip of the fourth toe and the base of the inner metatarsal tubercle (ToL4), distance between the tip of the first toe and the base of the outer metatarsal tubercle (ToL1), width of the hand at its widest point (HaW) and distance between the base of the hand and the tip of the third

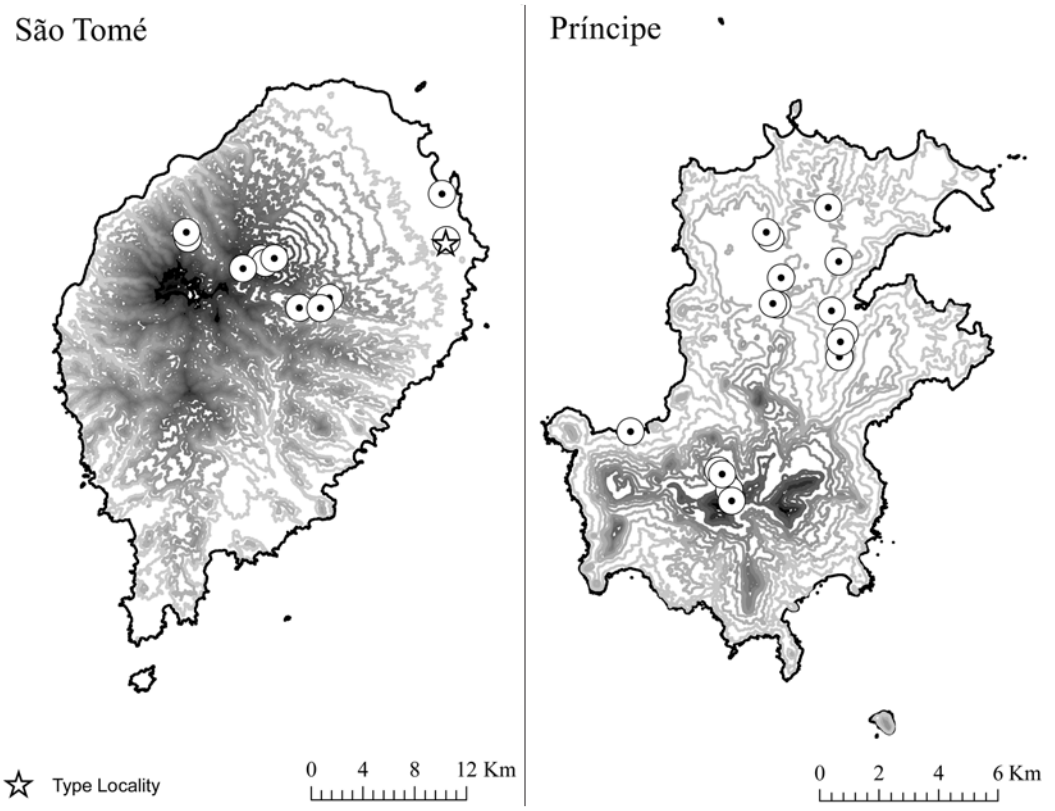

FIGURE 1. Collection localities from Príncipe and São Tomé. Type locality for *P. leveleve* on São Tomé is indicated.

finger (FiL3). We performed linear discriminant function analysis (DFA) of males for *P. dispar* and *P. leveleve* using ratios of 17 morphometric characters to SVL. For each island population, we randomly divided individuals into equal subgroups, only one of which was used to calculate the discriminant functions. The remaining individuals, as well as the two syntypes of *P. feae,* were reclassified using the discriminant function to determine reclassification scores.

OSTEOLOGY.— Two males and one female from each island were cleared and double-stained with alcian blue and alizarin red dyes, following the procedure of Dingerkuis and Uhler (1977), as modified by Drewes (1984). Specimens were examined using a dissecting microscope.

CYTOCHROME B SEQUENCES.— We extracted mitochondrial DNA from 17 ingroup specimens and one outgroup specimen (*Phrynobatrachus dendrobates,* Appendix Table 2) using a Qiagen DNeasy tissue extraction kit (Qiagen Inc, Valencia, CA, USA) following the manufacturer's recommendations for animal tissue. Cytochrome b sequences were amplified using the L-strand primer MVZ15 [5' GAA CTA ATG GCC CAC ACW WTA CGN AA 3'] (Moritz et al. 1992) and the H-strand primer Ptacek2-H [5' TCT TCT ACT GGT TGT CCT CCG ATT CA 3'] (Appendix Table 3, Ptacek et al. 1994). We performed hot start PCR reactions using 100 µL volumes with the top mix and bottom mix initially separated with a wax bead. The top mix (25 µL) consisted of 16 µL ddH_2O, 5 µL 10X Promega thermocycle buffer, 2 µL 10 mM dNTP, and 1 µL 25 µM of each primer. Bottom mix (75 µL) consisted of 0.5–20 µL of extracted DNA, 5 µL 10X Promega thermocycle buffer, 3–5 µL 50 mM $MgCl_2$, 1.5–2.5 µL Bioline Taq DNA Polymerase (Bioline, London, UK) and ddH_2O added to 75 µL. PCR reactions were carried out using a Perkin-Elmer 9600

Cetus PCR thermocycler. PCR product was verified using electrophoresis on a 0.8% agarose gel and staining with Ethidium Bromide. PCR product was purified using the Promega Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to manufacturer's recommendations. We subsequently cycle sequenced the PCR product (10 μL volumes) using 0.5–4 μL template, 1 μL 2.5 μM primer, 0.5 μL DMSO, 1 μL 5X Promega thermocycle buffer and 1 μL Big Dye v.3.1 reaction premix (Perkin-Elmer, Norwalk, CT, USA) filled to a total volume of 10 μL with ddH_2O. The cycle sequencing product was precipitated and washed with 100% ethanol, denatured with formamide and sequenced using an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Forward and reverse complement sequences were edited, aligned and checked visually for ambiguous base assignments using Sequencher 3.0 (Gene Codes Corporation). Sequences were aligned using ClustalX v.1.83 (Thompson et al. 1997). Sequences were protein-coding and thus alignment was unambiguous with no indels. A model of molecular evolution was selected from the data using Modeltest 3.7 using the corrected Akaike Information Criterion (Posada and Crandall 1998). Once a model was selected, the sequences were imported into PAUP* (Swofford 1999) and a maximum-likelihood (ML) tree constructed using a heuristic search with tree-bisection-reconnection (TBR) branch swapping with 10 random addition sequence replicates. Bootstrap support for the resulting nodes was estimated using 1000 ML bootstrap replicates with TBR branch swapping and 10 random addition sequence replicates (Felsenstein 1985). All unique sequences were deposited in Genbank (EUO 74967-984).

12S rRNA, tRNA-valine, and 16S rRNA Sequences.— DNA from 28 specimens (Appendix Table 2) was extracted and the mitochondrial 12S rRNA, valine-tRNA, and 16S rRNA regions were sequenced to obtain a fragment of about 2350 base pairs (Appendix Table 3). The fragment was amplified using four overlapping PCR products of approximately 600bp. Sequences were aligned using ClustalX and further refined utilizing MacClade 4.06. Maximum Parsimony analyses were carried out utilizing PAUP* 4.0b10 (Swofford 2002), using the heuristic search option with TBR branch swapping and 1000 random addition sequence replicates. A model of molecular evolution was selected from the data using Modeltest 3.7 using Hierarchical Likelihood Ration Tests (Posada and Crandall 1998). Once a model was selected, the sequences were imported into PAUP* (Swofford 1999) and a maximum-likelihood (ML) tree constructed using a heuristic search with tree-bisection-reconnection (TBR) branch swapping with 10 random addition sequence replicates. Bootstrap support for the resulting nodes was estimated using 100 ML bootstrap replicates with TBR branch swapping and 10 random addition sequence replicates (Felsenstein 1985). All unique sequences were deposited in Genbank (EUO 75275-302).

Species Description

Phrynobatrachus leveleve Uyeda, Drewes, and Zimkus, sp. nov.

Material examined.— Holotype: CAS 218901 male; São Tomé and Príncipe: São Tomé Island, Caxueira, along Agua Pete Pete, 0°18′N, 6°44′E, elevation 50 m. Collected by R.C. Drewes, R.E. Stoelting, and J.V. Vindum, 5 April 2001. Paratypes: CAS 218894, CAS 218892 male and female respectively and CAS 218895 (male, cleared and stained) collected from type locality (sequences from this specimen are also included in Frost et al., 2006). CAS 219003 male and CAS 218998 (female, cleared and stained), collected from Java 0°16′N, 6°39′E, elevation 600 m. CAS 219066 female; collected from the west side of the Rio Contador, 0°18′N, 6°33′E, elevation 700 m. All specimens collected between 2–15 April 2001 by R.C. Drewes, R.E. Stoelting, and J.V. Vindum. Other Material Examined: CAS 218893 female; CAS 218896–218900 males; from type locality. CAS 218906 male; on road between Bombaim and Santa Adelaide at Rio Abade bridge

0°15′N, 6°38′E, elevation 50 m. CAS 218918, 218919, 219064, 219065 females; CAS 219067 male; on west side of the Rio Contador, 0°18′N, 6°33′E, elevation 700 m. CAS 219406 female, CAS 218995–218997, 218999–219004, 219407–219409 males; Java, 0°16′N, 6°39′E, elevation 600 m. CAS 219027 male; Quisinda, 0°18′N, 6°44′E, elevation 50 m. CAS 219051 female; CAS 219052,219053 males; between Bom Sucesso and Lagoa Amelia, 0°17′N, 6°36′E, elevation 1100 m. CAS 219264–219268 males; city of São Tomé, 0°20′N, 6°43′E, elevation 0 m. CAS 219319–219321, males; Macambrara, 0°17′N, 6°36′E, elevation 1100 m. CAS 233677, 233680, 233685, 233688, 233698–233699 females; CAS 233678–233679, 233681–233684, 233686–233687, 233689–233691, 233704 males; vicinity of Abade, (0°20′N, 6°44′E), elevation 400 m. CAS 233700, juvenile; CAS 233701 female; Lagoa Amelia, (0°17′N, 6°35′E), elevation 1412 m.

Diagnosis.— Adult males distinguished from *Phrynobatrachus dispar* by a lower jaw distinctly marked with vertical banding, a darkened vocal sac, the presence of minute spicules arranged in a U-shaped pattern along the anterior margin of the jaw and a proportionally smaller eye (Figs. 2, 3). Dorsal asperities are never as distinct or extensive as those in male *P. dispar* (this difference is obvious even in subadult and recently metamorphosed specimens). Female *P. leveleve* are distinguished from female *P. dispar* by the absence of asperities in most individuals, smaller size and duller coloration (Fig. 4). Although highly polymorphic, the overall coloration of both male and female *P. leveleve* is duller, generally lacking distinct vertical barring on the thigh and leg as found in *P. dispar* (Fig. 2).

Phrynobatrachus leveleve is distinguished from *P. calcaratus* and *P. cornutus* by the absence of an eyelid cornicle (although a small bump may be observed in the same location). *P. leveleve* is further distinguished from *P. parvulus* and *P. minutus* of the mainland by larger size, stouter habitus and smaller femoral glands in males.

Etymology.— The specific epithet is derived from the native Portuguese Creole spoken in the Republic of São Tomé and Príncipe. The phrase, "*leve leve*," generally meaning "easy, easy" or "lightly lightly" has also been translated by Henrique Pinto da Costa, former Minister of Agriculture, as "calmly, surely." In our opinion, all three definitions describe the delightful, easy-going demeanor of the citizens of the Republic São Tomé and Príncipe. With the recent discovery of oil in the Gulf of Guinea, greed and exploitation threaten to disrupt the peaceful culture of São Tomé, and it is with the hope that the citizens of this tiny African nation will maintain their ecological heritage and cheerful outlook on life that we name this diminutive endemic anuran.

Description of the Holotype.— CAS 218901 Male, 15.5mm SVL; total length of the leg is 1.5–2.0 times the SVL. Width of the head greater than 2.5 times the diameter of the eye. Dorsal asperities are indistinct to the naked eye and are most numerous between the posterior half of the eyelid and the tibio-fibula. However, a few asperities extend beneath the eye and onto the snout as well as the anterior portion of the eyelid. Tympanum indistinct, less than half the width of the eye. Two indistinct glandular ridges topped with 3 to 4 white-pointed asperities forming a broken X-shaped pattern are located on the mid-dorsum.

The gular sac is darkened and appears as an inverted "U" shaped patch on the throat. The anterior border of the dark pigment contains numerous spicules that extend from the very tip of the lower jaw posterior to the corners of the mouth. These are found in 5–7 rows on the tip of the jaw and narrow to only 1–2 rows at the angle of the jaw. The medial lingual papilla on the tongue is present. Femoral glands, although indistinct, are present in the middle of the thigh; the glands extend about one-fourth the total length of the thigh.

Webbing between fingers absent, webbing between toes reduced and deeply incised, existing mostly as a narrow fringe on the sides of the toes, webbing formula I2-3II3^{-}–3^{+}III3^{+}-4^{+}IV5^{-}–4^{-}V (Savage and Heyer 1997). Distal phalanx T-shaped, resulting in the appearance of dilated toe tips.

Color in preservative.: Dorsum ground color a dark grayish-brown, lacking many distinctive markings. Two dark blotches over the front legs extend diagonally toward the eye. Faint light inter-

FIGURE 2. Comparison of adult males in life. *P. leveleve* (A.; from series CAS 218995-218003- Java) and *P. dispar* (B.; from series CAS 219080-219124 – Agua Doutor). Notice the more prominent and numerous asperities in *P. dispar* as well as the more striking coloration. Some dark barring on the lower jaw of *P. leveleve* is also visible.

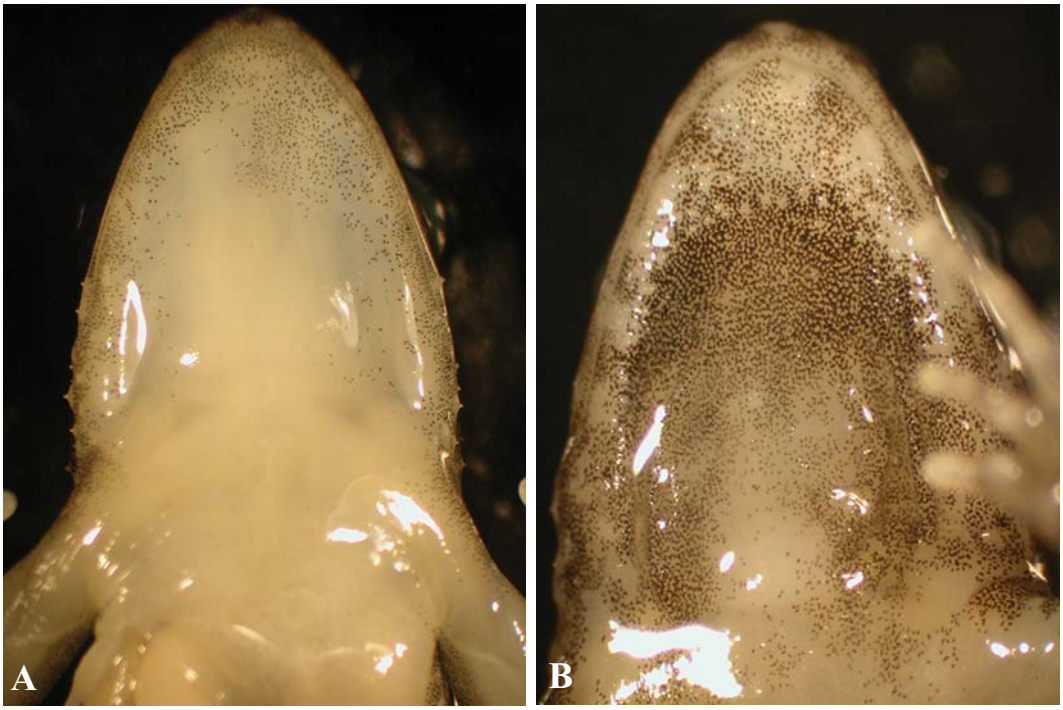

FIGURE 3. Comparison of the throat patterns in *P. dispar* (A) and *P. leveleve* (B). Note the presence of numerous spicules, darkened vocal sac and barring on the lower jaw of *P. leveleve* compared with the clear, cream colored throat of *P. dispar.*

orbital stripe followed by a faint dark patch in the center of the dorsum behind the eyes. Indistinct dark splotches are present on the dorsum. Only one or two faint dark bars are found on either the thigh or the tibio-fibula of the hind limb. Ventrum pale cream-colored and clear except for the throat and a few darkly pigmented spots extending along the flanks to just beyond the front legs. Seven distinct dark brown bars line the lower jaw. Undersides of the hind limbs clear, slight yellowish hue.

VARIATION IN THE PARATYPES.— Morphology of the paratypes is generally consistent with that of the holotype. CAS 218894 male: 14.5 mm SVL, specimen with a dark brown ground and a light mid-dorsal stripe present, approximately 0.2 mm wide, running from just anterior the eyes, split-

ting just before the cloaca into two stripes that run down the posterior side of the both thighs and tibio-fibulae ending at the tarsus. CAS 219003 male: 17.1 mm SVL, lighter brown ground color with distinct darker brown streaks in a broken "X" on the dorsum, above the forelimbs and underneath the tympanum. Dorsal asperities extend onto the snout and hind legs. CAS 219882 gravid female: 18.9 mm SVL, specimen is uniform brown with a grayish wash on the flanks. Ventrum cream colored, only a few flecks of brown, with strong barring on the lower lip. CAS 219066 gravid female: 20.5 mm SVL, specimen is uniform brown with a dark snout. Ventral surface marked with large dark splotches throughout.

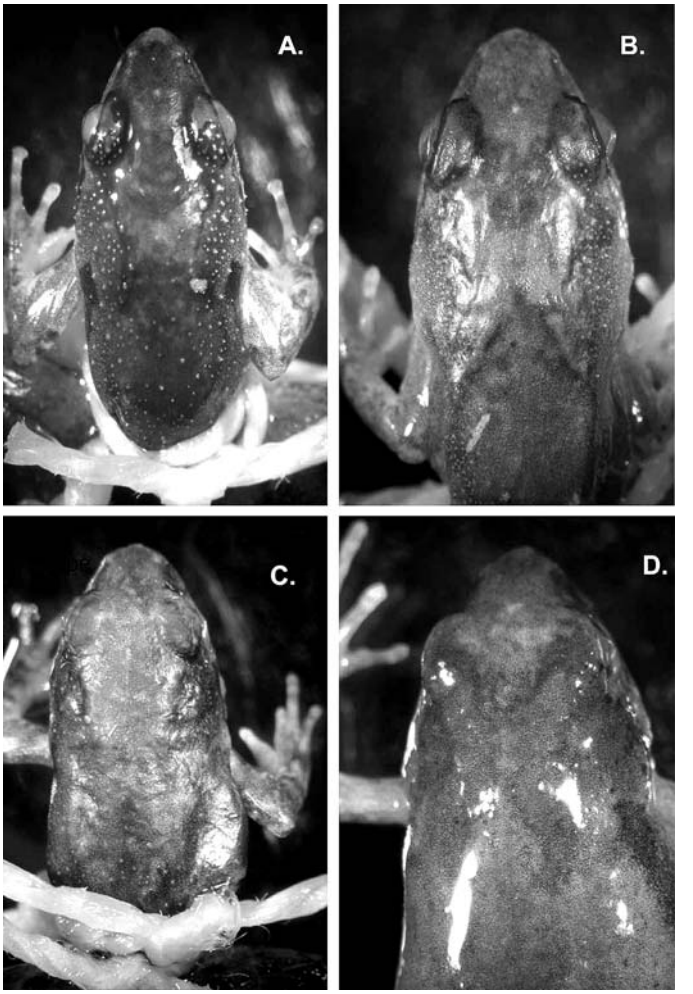

FIGURE 4. Comparison of dorsal asperities in *P. dispar* male (A) and female (C) with *P. leveleve* male (B) and female (D).

CYTOCHROME B SEQUENCE VARIATION.— We obtained sequences for seven individuals from four localities on Príncipe and eight individuals from three localities on São Tomé (Appendix Table 2). Outgroup sequences include a single sequence obtained for *P. dendrobates* as well as two sequences obtained from Genbank: *Rana catesbeiana* (AF205089) and *Rana nigromaculata* (AY315755). A segment of the cytochrome b gene 776 bp long was obtained for all individuals included in the analysis. Base frequencies were typical of vertebrate mitochondrial sequences [f(A) = 0.24, f(C) = 0.31, f(G) = 0.14, f(T) = 0.31]. Among ingroup taxa, there were 150 variable sites, 136 of which were parsimony informative. Modeltest selected a TVM+I+G model with a gamma distribution of 3.2151 and a proportion of invariable sites of 0.5128.

Intra-island pairwise comparisons were generally quite low. On Príncipe, sequence divergence ranged from 0.001–0.023 (mean = 0.010 ± 0.002) and on São Tomé, from 0.000–0.009 (mean = 0.004 ± 0.001). By contrast, inter-island pairwise comparisons were quite high, with a mean sequence divergence of 0.21 ± 0.02.

Phylogenetic reconstruction demonstrates monophyly of both *Phrynobatrachus dispar* from Príncipe and *P. leveleve* from São Tomé (Fig. 5). Intra-island sequence divergence was higher on Príncipe than on São Tomé. One individual from Príncipe, CAS 219202, shared 6 bases with São

Tomé sequences that were not found in any other frogs from Príncipe. In fact, no other frog from Príncipe shares more than one parsimony informative site with São Tomé frogs. This likely represents a plesiomorphic state (in CAS 219202), as 4 of 6 these sites share the same base as at least one of the outgroup taxa.

Bootstrap values were high supporting the monophyly of the two island clades apart from *P. dendrobates*. In addition, there are high bootstrap values for both island clades, although there is a considerably lower value for the Príncipe clade (bootstrap value = 76). This decreased value is again due to CAS 219202, which shares character states with São Tomé populations to the exclusion of the rest of the Príncipe clade. Furthermore, for *P. leveleve* to be nested within *P. dispar* it would require an unrealistic disparity in rates of mutation. In fact, the bootstrap value becomes 100 when a molecular clock is enforced.

12S rRNA, valine-tRNA, and 16S rRNA sequence variation.— Approximately 2.4 kb of mtDNA, including the12S rRNA, valine-tRNA, and 16S rRNA genes, was obtained for 28 individuals representing twelve species of *Phrynobatrachus* (Appendix Table 2). *Petropedetes newtoni* (MCZ136798) was included as the outgroup for this particular analysis. The maximum parsimony (MP) strict consensus and maximum likelihood (ML) tree resulted in fully compatible topologies. The MP analysis of 2,456 characters (960 variable, 762 parsimony informative) yielded one most parsimonious tree (Fig. 6). Base frequencies were typical of vertebrate mitochondrial sequences [f(A) = 0.34, f(C) = 0.23, f(G) = 0.18, f(T) = 0.25]. Modeltest selected a TrN+I+G model with a gamma distribution of 0.4126 and a proportion of invariable sites of 0.2794.

Phylogenetic reconstruction utilizing the12S rRNA, valine-tRNA, and 16S rRNA genes demonstrates monophyly of both *Phrynobatrachus dispar* from Príncipe and *P. leveleve* from São Tomé. Sequence divergence within species was generally trivial compared to among-species divergences with inter-island pair-wise comparisons having a mean sequence divergence of 0.057 ± 0.002. Intra-island sequence divergence was slightly higher on Príncipe than on São Tomé. Sequence divergence of *Phrynobatrachus* from Príncipe ranged from 0.001–0.005 (mean = 0.003 ± 0.001), whereas on São Tomé the divergence ranged from 0.001–0.003 (mean = 0.002 ± 0.001) (Fig. 6).

There are high ML bootstrap values for each island clade, supporting the monophyly of both *Phrynobatrachus dispar* and *P. leveleve*. In addition, bootstrap values of the clade containing these two sister species were high, supporting the two island species apart from other East African *Phrynobtrachus*. Lastly, the monophyletic group including *Phrynobatrachus dispar*, P. *leveleve*, and a group of East African species (*P. keniensis*, *P. inexpectatus*, *P. cf. minutus*, *P. parvulus*, and *P. rungwensis*) was found have high bootstrap values (Figs. 5, 6).

Morphological Variation.— Intra-island variation: Both *Phrynobatrachus dispar* and *P. leveleve* exhibit strong sexual dimorphism. Male *P. dispar* are distinguished by greater density and size of dorsal asperities, smaller size and the presence of nuptial pads. Female *P. dispar* also have dorsal asperities, but these are much smaller, more numerous, and located predominantly on the flanks. In *P. leveleve*, males have small and sparsely distributed dorsal asperities, a darkened vocal sac and small spicules on the underside of the throat. Most female *P. leveleve* lack dorsal asperities entirely, and extremely small and sparse asperities were only observed in a single individual (CAS 233677).

In general, within island morphology was consistent on both São Tomé and Príncipe. Furthermore, we found no evidence for the bimodal distribution of tubercle distance ratios (ITT/IOMT) that Boulenger used to distinguish *Phrynobatrachus feae*. This measurement often varied in a single individual, depending on whether the right or left foot was used. Although larger individuals were often found at higher elevations, this variation appears to be continuous rather than discrete

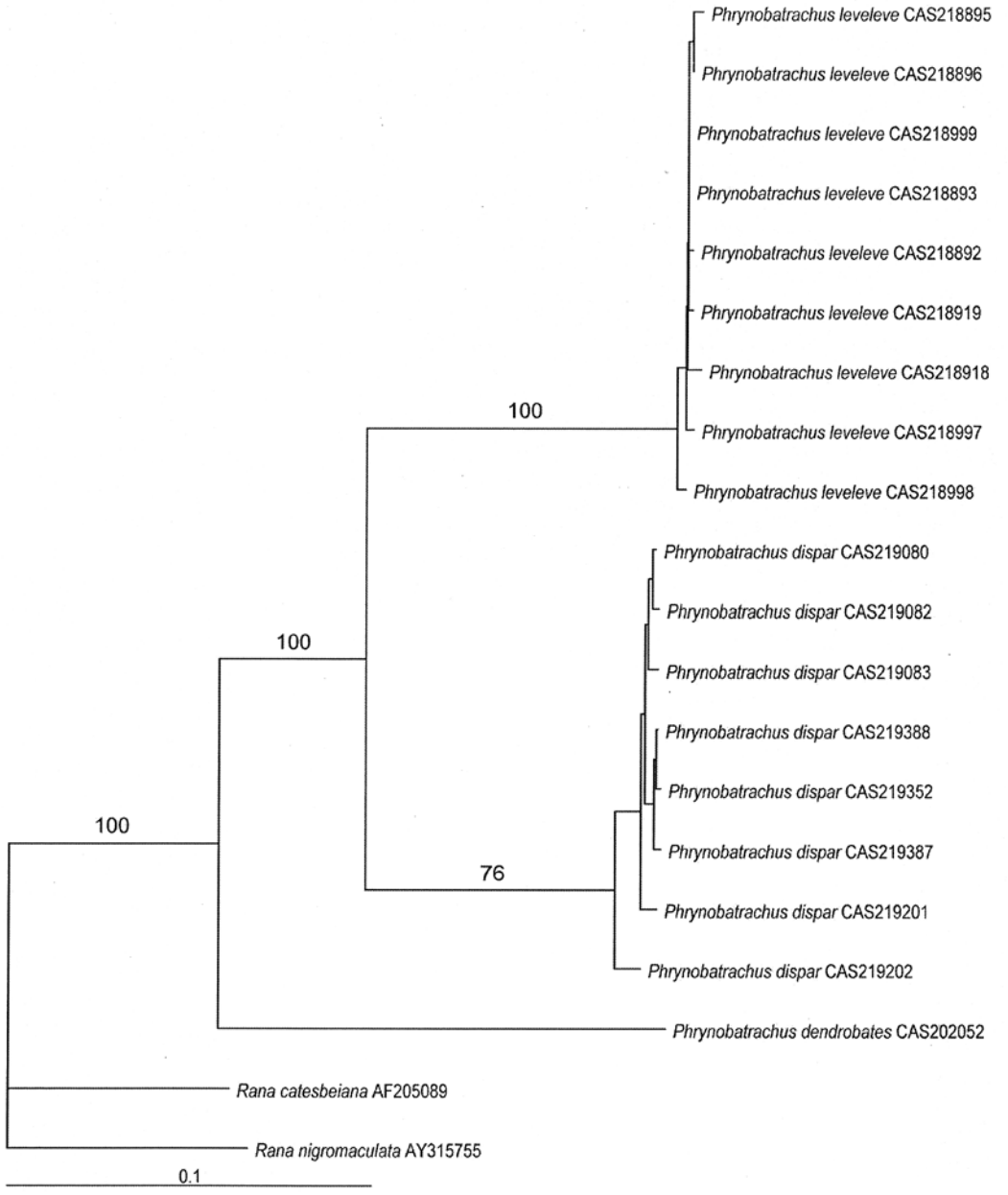

FIGURE 5. Maximum likelihood phylogram of cytochrome b gene with bootstrap support indicated at the nodes. Note the large sequence divergence between *P. dispar* from Príncipe and *P. leveleve* from São Tomé.

(Fig. 7). Thus, it appears that Baillie's observation that two distinct size classes in different habitats may have actually been an observation of Bergmann's rule across an elevational gradient (Ashton 2002). Both São Tomé and Príncipe rise steeply out of the ocean, and it is easy to imagine mistaking continuous variation based upon elevation for discrete size classes. Interestingly, this phenomenon has also been observed in the island's endemic caecilian, *Schistometopum thomense*,

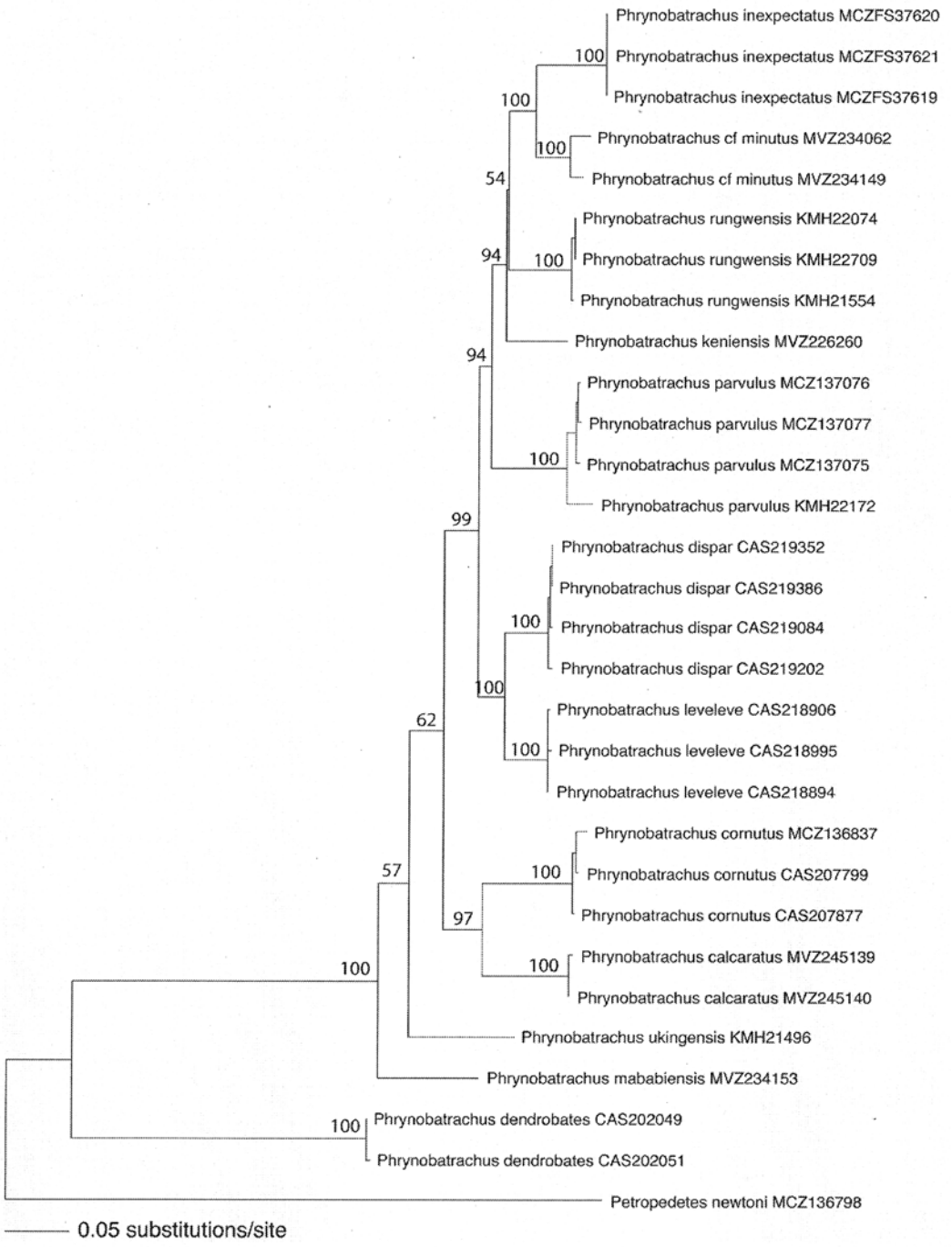

FIGURE 6. Maximum likelihood phylogeny of combined 12S rRNA, valine t-RNA, and 16S rRNA of 12 species of *Phrynobatrachus.* Bootstrap values are indicated at the nodes.

which reaches its greatest size at higher elevations (Measey and Van Dongen 2006).

INTER-ISLAND VARIATION: *Phrynobatrachus dispar* and *P. leveleve* can be readily distinguished from each other in both sexes. Males of *P. leveleve* have fewer asperities, which are only faintly noticeable to the naked eye. By contrast, *P. dispar* males have distinct white-tipped conical asperities (Figs. 2 and 3). Consistent with Baillie's observation (1999) that there are more color morphs on Príncipe than on São Tomé, the dorsal coloration of *P. dispar* is more striking than that of *P. leveleve*, which is most often a drab grayish brown in alcohol. Dark barring on the legs is also more distinct in *P. dispar* than *P. leveleve* (Fig. 2). Ventral coloration follows the opposite pattern. Male *P. dispar* usually have a clear, cream colored throat whereas male *P. leveleve* have dark barring on the lower jaw, a darkened vocal sac and minute spicules along its anterior margin. As noted by Frost et al (2006), the presence of spicules on the throat appears sporadically within the Phrynobatrachidae and Petropedetidae, in taxa as morphologically divergent as *Conraua* and *Phrynobatrachus*, yet may be absent in a sister species as observed in the Gulf of Guinea *Phrynobatrachus*.

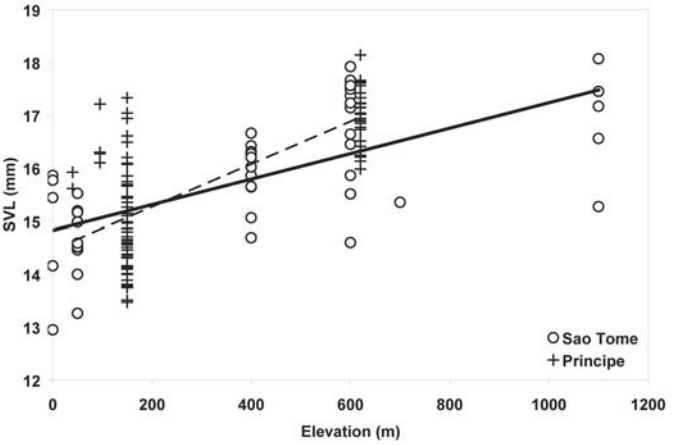

FIGURE 7. Increasing snout-vent length (SVL) with increasing elevation for adult males from Príncipe and São Tomé. Solid line is the regression line for São Tomé, dashed line is for Principe. Regression lines for both islands have highly significant non-zero slopes ($p < 0.01$).

Female *Phrynobatrachus dispar* are distinguished from female *P. leveleve* by the presence of numerous minute asperities on the flanks of the body (Fig. 4). Ventral coloration varies from large, distinct brown blotches against a cream colored background to diffuse mottling of light brown spots and various combinations thereof. Individuals of both species exhibit varying degrees of dark barring on the lower jaw.

The two species are remarkably similar morphometrically given the high values obtained for sequence divergence (Appendix Table 4). Females from Príncipe were significantly larger ($N = 20$, mean SVL = 22.2 mm) than females from São Tomé ($N = 17$, SVL = 19.6), even though females from São Tomé were collected, on average, at higher elevations than individuals from Príncipe. For inter-island comparisons between males, EYE, ENS, HaW, NLL, and FiL3 differed significantly (2-tailed student's t-test, $p < 0.05$), as well as the ratios in both sexes for EYE/NLL (males and females, $p < 0.01$), EYE/HW (males, $p < 0.01$; females, $p < 0.05$) and HaW/FiL3 (males and females, $p < 0.01$) (Appendix Table 4). In particular, the size of the eye in *P. dispar* is noticeably larger than in *P. leveleve.* Although there is some overlap in this character, the maximum measurement in *Phrynobatrachus leveleve* is less than the mean for *P. dispar* in both males and females.

Discriminant function analysis indicated reliable patterns of character variation for males of the ingroup taxa, although some overlap did occur. A second DFA using only a random subset of each of the ingroup species weighted loadings similarly to the first, with the highest absolute loadings for the ratios of EYE, NLL, and ENS to SVL. Furthermore, of those individuals that were not included in the smaller sample DFA, 86% of *Phrynobatrachus dispar* ($N = 26$) and 81% of *P. leveleve* ($N = 16$) were correctly identified to species. Classification of the two *P. feae* type specimens

examined was split, with one being classified as *P. dispar* and one being classified as *P. leveleve* by the discriminant functions. This is not surprising inasmuch as the data used to train the discriminant functions are based primarily on measurements of adults, whereas the types of *P. feae* appear to be juveniles. Combined with the morphological homogeneity and low sequence divergence values for intra-island comparisons, we conclude that *P. feae* is a junior synonym of *P. dispar*.

Internal morphology is similar in all six individuals examined, suggesting that these two species are closely related. Some differences in skull morphology were observed between *Phrynobatrachus dispar* and *P. leveleve*. The anterior margin of the frontoparietal in *P. dispar* is deeply incised, whereas in *P. leveleve* it is only slightly irregular, and the spacing between the nasal cartilage appears greater in *P. dispar* than in *P. leveleve* (Fig. 8), although this character was variable in our small sample size.

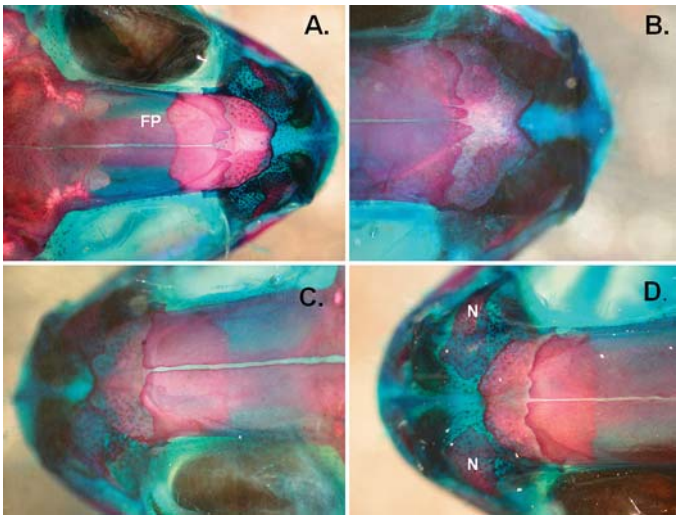

FIGURE 8. Snout of cleared and double-stained specimens of *P. dispar* male (A), female (B), and *P. leveleve* male (C) and female (D). Notice the anterior margin of the frontoparietal (FP) in *P. dispar* is more deeply incised than that of *P. leveleve*. Also the spacing between the nasals (N) is greater in the former.

DISCUSSION

Although we find no evidence for the existence of two taxa on Príncipe, it is intriguing that Boulenger describes *Phrynobatrachus feae* as having a throat that is dark brown or black, with uniform or round white spots (Boulenger 1906), which is a character we use to distinguish *P. leveleve*. All adult material we examined from Príncipe possessed a clear white throat. It is possible that Boulenger's specimens, collected by L. Fea, may have been from São Tomé, but there are no existing records at the British Museum that suggest the specimens were collected elsewhere than Príncipe ("Prince's Island") (B.T. Clarke, pers. comm.). Furthermore, the syntypes of *P. feae* examined (BMNH 1947.2.6.89, 91) appear to be juvenile *P. dispar*. Juvenile specimens tend to be darker overall and often have completely dark throats that fade at maturity. Although the coloration and presence of asperities on the syntypes were difficult to determine because of their state of preservation, we could find no reason to doubt that these are juvenile *P. dispar* collected from Príncipe Island.

Sequence data demonstrates considerable divergence between the two island species of *Phrynobatrachus*. Using a low estimate of divergence of 19% for the cytochrome b gene and a molecular clock estimate as high as 1.4% sequence divergence per million years, a value considerably higher than estimated divergence rates found in other amphibians (Caccone et al. 1997; Veith et al. 2003), suggests a time of divergence that predates the estimated origin for São Tomé of 13 million years ago (Lee et al. 1994). This would seem to suggest that the most likely scenario for the divergence of the two species would be independent colonizations from mainland Africa. After the recognition into two *Phrynobatrachus* species, each endemic to a single island, only one species of amphibian, *Hyperolius molleri,* appears to have successfully dispersed from one island to the other (or recently colonized both islands). However, it is difficult to make conclusions with

any certainty given the unreliability of molecular clock estimates, the uncertainty of the sequence divergence, and the possibility that the estimated age of São Tomé may actually be much greater than 13 million years (orogeny dates were based upon the oldest lava flows, which only set a minimum age for the island). Analyses of 12S rRNA, valine-tRNA, and 16S rRNA mtDNA further support the divergence between the two island species of *Phrynobatrachus* as intra-island variability was low in comparison to interspecific divergence.

Surprisingly, a number of recent molecular studies suggest that many of the endemic amphibians of the Gulf of Guinea islands may be more closely related to East African species than to their West African congeners; these studies include the fossorial caecilian, *Schistometopum thomense* (Wilkinson et al. 2003), *Ptychadena newtoni* (Measey et al. 2007) and, perhaps the treefrogs, *Hyperolius molleri* and *H. thomensis.* (Drewes and Wilkinson 2004, show the closest outgroup relative to be *H. cinnamomeoventris,* whose range includes East Africa). Similar claims have been made for the endemic terrestrial gastropod mollusk, *Bocageia* (Gascoigne 1994). Whereas this pattern may be a reflection of poor sampling in the intervening Congo Basin, the inclusion of the larger dataset (BMZ) in this study (12 *Phrynobatrachus* species) seems to support a Gulf of Guinea island-East Africa relationship (Fig. 6). *Phrynobatrachus dispar* and *P. leveleve* consistently form a clade with *Phrynobatrachus* species from Ethiopia, Kenya, Malawi, and Tanzania (*P. keniensis*, *P. inexpectatus*, *P.* cf. *minutus*, *P. parvulus*, and *P. rungwensis*), rather than with the West African species *P. calcaratus* and *P. cornutus*. This curious pattern draws attention to the need for phylogenetic reconstruction of the Gulf of Guinea amphibians within the context of the African amphibian fauna, which may provide considerable data on the mechanisms and timing of colonization events. Currently such studies are limited by poor collections across Africa, especially the Congo Basin, and a paucity of researchers with an interest in African herpetofauna. We call attention to this deficit in the hopes that future researchers will take on the challenge of providing a comprehensive treatment of these species.

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Appendix

TABLE 1. Localities for all specimens for which morphological data were collected. Additional information is available online in the CAS catalog: <*http://www.calacademy.org/research/herpetology/catalog/*>

Species	Catalog Number	Elev.	Locality	Lat. and Long.
P. leveleve	CAS 218892-6218901	50 m	São Tomé	0°18′N, 6°44′E
	CAS 218906	50 m		0°15′N, 6°38′E
	CAS 218918-218919, 219064-219067	700 m		0°18′N, 6°33′E
	CAS 218995-219003, 219406-219409, 219004	600 m		0°16′N, 6°39′E
	CAS 219027	50 m		0°18′N, 6°44′E
	CAS 219051-219053	1100 m		0°17′N, 6°36′E
	CAS 219264-219269	0 m		0°20′N, 6°43′E
	CAS 219319-219321	1100 m		0°17′N, 6°36′E
	CAS 233677-233691, 233704	400 m		
	CAS 233700-233701	1412 m		
P. dispar	CAS 219080-219124	150 m	Príncipe	1°39′N, 7°25′E
	CAS 219143-219147	150 m		1°40′N, 7°25′E
	CAS 219201-219202	0 m		1°36′N, 7°21′E
	CAS 219211	0 m		
	CAS 219345-219346	175 m		1°39′N, 7°24′E
	CAS 219352-219356	150 m		1°38′N, 7°24′E
	CAS 219363	50 m		1°38′N, 7°25′E
	CAS 219385-219392, 233535-233567	620 m		1°35′N, 7°23′E
	CAS 219393-219394, 233569	948 m		1°34′N, 7°23′E
	CAS 233527-233530, 233533-233534	40-95 m		
"*P. feae*"	BMNH.1947.2.6.89, 91*		Príncipe	
P. cornutus	CAS 207877-207880, 207885, 207890, 207897-207901		Bioko Id	3º19′N, 8º40′E
P. calcaratus	CAS 199267-199299		Cameroon	3º11′N, 12º49′E
P. parvulus	CAS 204580-204592		Uganda	
P. cf. *minutus*	CAS 122939-122964		Marsabit, Kenya	

* Syntypes

TABLE 2. Specimens included in the two molecular datasets.

Species	Collection no.	Locality	Genbank acc#
16S rRNA, 12S rRNA and tRNA Valine			
Phrynobatrachus dispar	CAS219084	Príncipe	EU075275
	CAS219202	Príncipe	EU075276
	CAS219352	Príncipe	EU075277
	CAS219386	Príncipe	EU075278
P. leveleve	CAS218894*	São Tomé	EU075279
	CAS218906	São Tomé	EU075280
	CAS218995*	São Tomé	DQ283223
P. calcaratus	MVZ245139	Ghana	EU075281
	MVZ245140	Ghana	EU075282
P. cornutus	CAS207799	Bioko	EU075283
	CAS207877	Bioko	EU075284
	MCZ136837	Cameroon	EU075285
P. dendrobates	CAS202049	Uganda	EU075286
	CAS202051	Uganda	EU075287
P. cf. minutus	MVZ234062	Kenya	EU075288
	MVZ234149	Kenya	EU075289
P. inexpectatus	MCZFS37619	Ethiopia	EU075290
	MCZFS37620	Ethiopia	EU075291
	MCZFS37621	Ethiopia	EU075292
P. keniensis	MVZ226260	Kenya	EU075293
P. mababiensis	MVZ234153	Kenya	EU075294
P. parvulus	MCZ137075	Malawi	EU075295
	MCZ137076	Malawi	EU075296
	MCZ137077	Malawi	EU075297
P. rungwensis	KMH21554	Tanzania	EU075298
	KMH22074	Tanzania	EU075299
	KMH22709	Tanzania	EU075300
P. ukingensis	KMH21496	Tanzania	EU075301
Petropedetes newtoni	MCZ136798	Cameroon	EU075302
Cytochrome b			
Phrynobatrachus dispar	CAS219201	Príncipe	EU074967
	CAS219202	Príncipe	EU074974
	CAS219080	Príncipe	EU074968
	CAS219082	Príncipe	EU074969
	CAS219083	Príncipe	EU074973
	CAS219352	Príncipe	EU074972
	CAS219387	Príncipe	EU074970
	CAS219388	Príncipe	EU074971
P. leveleve	CAS218997	São Tomé	EU074980
	CAS218998*	São Tomé	EU074977
	CAS218999	São Tomé	EU074978
	CAS218918	São Tomé	EU074983
	CAS218919	São Tomé	EU074979
	CAS218892	São Tomé	EU074976
	CAS218893	São Tomé	EU074982
	CAS218895	São Tomé	EU074975
	CAS218896	São Tomé	EU074981
P. dendrobates	CAS202052	Uganda	EU074984
Rana catesbeiana		USA	AF205089
R. nigromaculata		Korea	AY315755

Paratypes *

TABLE 3. Primers used to amplify cytochrome b, 12S rRNA, tRNA valine, and 16S rRNA genes

Primer Name	Designation (1)	Position*
MVZ15-L	141 MVZ15-L	16243-16268
Ptacek2-H	168 Ptacek2-H	17257-17282
MVZ59-L	29 MVZ59	2153-2180
MVZ59B-L	————	2236-2263
12L1-L	46 L1091	2475-2509
12SM-L	————	2968-2988
tRNAval-H	73 tRNAval-H	3034-3059
16SH-H	————	3282-3304
16SC-L	————	3623-3642
16SA-H	88 16Sar-H	3956-3975
16SD-H	96 16Sbr-H	4549-4574

*Positions relative to *Xenopus laevis* mitochondrial genome
1. Goebel, A.M., et al. 1999. *Molecular Phylogenetics and Evolution* 11(1):163-199.

TABLE 4. Male and female morphometric data for all adult ingroup specimens examined. Significant values t-test values for both interisland, same sex comparisons are indicated by an asterisk (*) (two-tailed, $p < 0.05$). See methods for abbreviations. Measurements: Range; mean ± S.D.

	P. dispar males (*n*=84)	*P. dispar* females (*n*=20)	*P. leveleve* males (*n*=44)	*P. leveleve* females (*n*=17)
SVL	13.5-18.1; 15.6 ± 1.3	17.8-24.7; 22.2 ± 1.9	13.0-18.1; 15.8 ± 1.2	18.1-21.4; 19.6 ± 1.0
HDL	5.9-8.1; 6.8 ± 0.5	7.1-10.1; 9.3 ± 0.8	5.8-8.0; 6.8 ± 0.5	7.3-8.5; 8.1 ± 0.4
HDW	4.8-6.4; 5.6 ± 0.4	6.2-9.1; 8.1 ± 0.7	4.5-6.2; 5.5 ± 0.4	6.1-7.5; 6.7 ± 0.4
EYE*	1.9-2.7; 2.3 ± 0.2	2.4-3.5; 3.0 ± 0.3	1.8-2.3; 2.0 ± 0.1	2.2-2.8; 2.4 ± 0.1
IOS	1.4-2.1; 1.8 ± 0.2	1.8-3.0; 2.4 ± 0.2	1.2-2.1; 1.7 ± 0.2	1.4-2.4; 2.1 ± 0.3
INS	1.7-2.2; 1.9 ± 0.1	2.1-3.0; 2.6 ± 0.2	1.6-2.2; 1.9 ± 0.2	1.9-2.5; 2.2 ± 0.1
ENS*	1.3-1.8; 1.5 ± 0.1	1.4-2.5; 2.0 ± 0.2	1.0-1.6; 1.3 ± 0.1	1.1-1.9; 1.7 ± 0.2
NS	0.9-1.5; 1.1 ± 0.1	1.1-1.9; 1.6 ± 0.2	0.6-1.4; 1.1 ± 0.1	1.2-1.6; 1.3 ± 0.1
NLL*	0.8-1.3; 1.0 ± 0.1	1.0-1.6; 1.3 ± 0.1	0.8-1.2; 1.0 ± 0.1	1.1-1.4; 1.2 ± 0.1
TiL	7.3-9.8; 8.4 ± 0.6	8.9-13.2; 11.9 ± 1.2	6.6-9.4; 8.3 ± 0.7	8.4-11.3; 10.3 ± 0.7
FeL	7.0-9.3; 8.0 ± 0.6	8.4-12.7; 11.2 ± 1.2	6.0-9.0; 7.7 ± 0.7	8.1-10.8; 9.7 ± 0.8
TaL	4.1-5.6; 4.7 ± 0.3	4.9-7.1; 6.5 ± 0.6	3.7-5.3; 4.6 ± 0.4	4.6-6.4; 5.7 ± 0.4
IOMT	0.5-0.9; 0.7 ± 0.1	0.7-1.2; 1.0 ± 0.2	0.3-1.0; 0.7 ± 0.2	0.4-1.2; 0.8 ± 0.2
ITT	0.7-1.4; 1.0 ± 0.2	0.9-1.9; 1.4 ± 0.3	0.4-1.2; 0.9 ± 0.2	0.7-1.6; 1.1 ± 0.2
ToL4	7.0-9.6; 8.3 ± 0.6	9.2-13.0; 11.7 ± 1.1	6.3-10.0; 8.3 ± 0.8	8.5-11.6; 10.3 ± 0.8
ToL1	1.8-2.8; 2.2 ± 0.2	2.4-3.8; 3.2 ± 0.4	1.7-2.9; 2.2 ± 0.3	2.2-3.2; 2.8 ± 0.3
HaW*	1.2-2.2, 1.6 ± 0.2	1.5-2.2; 1.9 ± 0.2	1.1-1.8; 1.5 ± 0.2	1.2-1.9; 1.6 ± 0.2
FiL3*	3.1-4.6; 3.9 ± 0.3	4.3-6.1; 5.4 ± 0.5	3.2-4.7; 4.0 ± 0.3	4.3-5.4; 4.8 ± 0.3
ENS/NLL*	1.3-1.8; 1.6 ± 0.1	1.3-1.9; 1.5 ± 0.1	1.1-1.6; 1.3 ± 0.1	0.9-1.6; 1.3 ± 0.1
EYE/NLL*	2.0-2.9; 2.4 ± 0.2	2.1-2.6; 2.3 ± 0.2	1.7-2.4; 2.1 ± 0.2	1.7-2.3; 1.9 ± 0.1
HW/EYE*	2.1-2.8; 2.4 ± 0.1	2.4-3.0; 2.7 ± 0.2	2.4-3.0; 2.7 ± 0.2	2.5-3.1; 2.8 ± 0.2
FiL3/HaW	1.9-3.0; 2.4 ± 0.2	2.5-3.3; 2.9 ± 0.3	2.2-3.7; 2.6 ± 0.3	2.6-3.8; 3.0 ± 0.3
ITT/IOMT	1.0-2.0; 1.4 ± 0.2	1.0-2.1; 1.5 ± 0.3	0.9-2.4; 1.3 ± 0.3	0.8-2.0; 1.4 ± 0.3